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Anti-Platelet Factor 4 Antibodies Causing VITT do not Cross-React with SARS-CoV-2 Spike Protein

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Abstract:
Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a severe adverse effect of ChAdOx1 nCoV-19 COVID-19 vaccine (Vaxzevria) and COVID-19 vaccine Janssen (Ad26.COV2.S), and associated with unusual thrombosis. VITT is caused by anti-platelet factor 4 (PF4) antibodies activating platelets through their FcgRIIa receptors. Antibodies activating platelets through FcgRIIa receptors have also been identified in COVID-19 patients. These findings raise concern that vaccination-induced antibodies against anti-SARS-CoV-2 spike protein cause thrombosis by cross-reacting with PF4. Immunogenic epitopes of PF4 and SARS-CoV-2 spike protein were compared using in-silico prediction tools and 3D-modelling. The SARS-CoV-2 spike protein and PF4 share at least one similar epitope. Reactivity of purified anti-PF4 antibodies from patients with VITT was tested against recombinant SARS-CoV-2 spike protein. However, none of the affinity-purified anti-PF4 antibodies from 14 VITT patients cross-reacted with SARS-CoV-2 spike protein. Sera from 222 PCR-confirmed COVID-19 patients from five European centers were tested by PF4/heparin ELISA and PF4-dependent platelet activation assays. We found anti-PF4 antibodies in 19 of 222 (8.6%) COVID-19 patient sera. However, only four showed weak to moderate platelet activation in the presence of PF4, and none of these patients developed thrombotic complications. Among 10 of 222 (4.5%) COVID-19 patients with thrombosis, none showed PF4-dependent platelet-activating antibodies. In conclusion, antibodies against PF4 induced by vaccination do not cross-react with the SARS-CoV-2 spike protein, indicating that the intended vaccine-induced immune response against SARS-CoV-2 spike protein is not the trigger of VITT. PF4-reactive antibodies found in COVID-19 patients of the present study were not associated with thrombotic complications.

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Main point 1: The immune responses to PF4 and to the spike protein are independent of one another

Main point 2: Antibodies from patients with vaccine-induced thrombocytopenia and thrombosis do not cross-react with the spike protein

Abstract

Vaccine-induced immune thrombotic thrombocytopenia (VITT) is a severe adverse effect of ChAdOx1 nCoV-19 COVID-19 vaccine (Vaxzevria) and COVID-19 vaccine Janssen (Ad26.COV2.S), and associated with unusual thrombosis. VITT is caused by anti-platelet factor 4 (PF4) antibodies activating platelets through their FcγRIIa receptors. Antibodies activating platelets through FcγRIIa receptors have also been identified in COVID-19 patients. These findings raise concern that vaccination-induced antibodies against anti-SARS-CoV-2 spike protein cause thrombosis by cross-reacting with PF4. Immunogenic epitopes of PF4 and SARS-CoV-2 spike protein were compared using in-silico prediction tools and 3D-modelling. The SARS-CoV-2 spike protein and PF4 share at least one similar epitope. Reactivity of purified anti-PF4 antibodies from patients with VITT was tested against recombinant SARS-CoV-2 spike protein. However, none of the affinity-purified anti-PF4 antibodies from 14 VITT patients cross-reacted with SARS-CoV-2 spike protein. Sera from 222 PCR-confirmed COVID-19 patients from five European centers were tested by PF4/heparin ELISA and PF4-dependent platelet activation assays. We found anti-PF4 antibodies in 19 of 222 (8.6%) COVID-19 patient sera. However, only four showed weak to moderate platelet activation in the presence of PF4, and none of these patients developed thrombotic complications. Among 10 of 222 (4.5%) COVID-19 patients with thrombosis, none showed PF4-dependent platelet-activating antibodies. In conclusion, antibodies against PF4 induced by vaccination do not cross-react with the SARS-CoV-2 spike protein, indicating that the intended vaccine-induced immune response against SARS-CoV-2 spike protein is not the trigger of VITT. PF4-reactive antibodies found in COVID-19 patients of the present study were not associated with thrombotic complications.

Introduction

Coronavirus disease 2019 (COVID-19) is caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), a single-stranded RNA virus, encoding 16 non-structural proteins (NSP's 1-16), 8 accessory proteins (ORF3a, 6, 7a, 7b, 8, 9b, 9c and 10) and 4 structural proteins, known as S (spike), E (envelope), M (membrane), and N (nucleocapsid) proteins.[1] The spike glycoprotein is responsible for recognition of host cell membrane receptors, ACE2, and TMPRSS2 and for mediating fusion with the host cell membrane.[2]

The European Medical Agency has approved four vaccines[3] for prevention of symptomatic COVID-19: two mRNA-based vaccines encoding the spike protein antigen of SARS-CoV-2, encapsulated in lipid nanoparticles, Comirnaty (BioNTech/Pfizer) and COVID-19 mRNA-1273 Vaccine Moderna; a recombinant chimpanzee adenoviral (ChAdOx1-S) vector encoding the spike glycoprotein of SARS-CoV-2, ChAdOx1 nCoV-19 COVID-19 vaccine (Vaxzevria; AstraZeneca); and a recombinant adenovirus type 26 vector encoding SARS-CoV-2 spike glycoprotein, COVID-19 Vaccine Janssen. Since March 2021, in Germany about hundred patients[4] with venous thromboses at unusual sites such as cerebral venous sinus thrombosis (CVT) and splanchnic vein thrombosis in combination with moderate to severe thrombocytopenia were observed in individuals approximately 5 to 30 days following vaccination with ChAdOx1 nCoV-19 COVID-19 Vaccine.[5-7] Similar complications have also been reported after vaccination with the COVID-19 Vaccine Janssen (Ad26.COV2.S).[8,9] Known as vaccine-induced immune thrombotic thrombocytopenia (VITT),[10] we have identified immunoglobulin G class platelet-activating antibodies directed against the cationic platelet chemokine, platelet factor 4 (PF4; CXCL4), as the underlying cause.[5]

Thromboembolic complications are also a major disease burden in hospitalized patients with COVID-19 disease, even in patients without severe respiratory disease. Sometimes thrombosis in COVID-19 patients also occurs at unusual locations such as cerebral veins.[11-13] However, the overall presentations of the COVID-19 patients and VITT patients are quite different. VITT patients often show laboratory signs of disseminated intravascular coagulation with severe thrombocytopenia and were otherwise well before the abrupt onset of thrombosis. In contrast, COVID-19 patients show disseminated intravascular coagulation typically only with severe disease or as a complication of extracorporeal circulatory support.[14]

A further similarity in VITT and COVID-19 patients is IgG-mediated platelet activation via platelet FcγIIa receptors. This has been shown for VITT patients[5] and also by two recent studies of COVID-19 patients.[15,16] Further, VITT patient sera usually react strongly in PF4/heparin ELISAs, a finding also seen in occasional patients with COVID-19.[17] But in contrast to the strong platelet-activating anti-PF4 antibodies from VITT patients, COVID-19 sera with anti-PF4 antibodies are usually non-platelet-activating.[17]

This overlapping clinical picture of unusual thrombotic complications, antibody-induced FcγIIa receptor-dependent platelet activation, and occasional reports of anti-PF4 antibodies in COVID-19 patients, raises the question as to whether the immune response against the spike protein induced by vaccination could induce antibodies that cross-react with immunogenic epitopes shared between spike protein and PF4. Accordingly, the overall aim of this study was to determine whether platelet-activating anti-PF4 antibodies in VITT patients cross-react with the spike protein, or whether the anti-PF4 and anti-spike immune responses are distinct. We addressed this by i) determining by structure analyses in silico whether there are shared immunogenic epitopes between SARS-CoV-2 spike protein and PF4, and ii) by classic immunohematology techniques of immunoadsorption to assess for cross-reactivity of anti-PF4 antibodies obtained from VITT patients against the spike protein. Our study also examined the presence of VITT-like anti-PF4 antibodies in patients with COVID-19 and their association with thrombosis.

Material and Methods

Identification of immunogenic epitopes and homologies of human PF4 and SARS-CoV-2 spike protein and comparative analysis of their 3D structures

The protein sequence for human PF4/CXCL4 was retrieved from the ENSEMBL gene data base (ENSG00000163737).[18] Similarly, the protein sequence of the SARS-CoV-2 spike protein (1273 amino acids) was retrieved from publicly available data bases (NCBI: Gene ID 43740568).[19] Using the online prediction tool of the University of Madrid, Spain (http://imed.med.ucm.es/Tools/antigenic.pl),[20] we identified potential immunogenic peptide sequences (epitopes) in both protein sequences. We used the SIM Alignment online Tool[21] and the MacMYPOL program,[22] together with the files 6vxx.pbd, 4r9w.pbd and 4hsv.pbd available from the PBD database[23] to compare the epitopes on the published structures of these three proteins.

VITT Patients

Sera from 24 VITT patients, defined as patients presenting with thrombocytopenia and thromboembolic events approximately 5-30 days after ChAdOx1 nCoV-19 COVID-19 vaccination with positive PF4/heparin ELISA and PF4 dependent platelet-activating antibodies tested in the Greifswald laboratory, were available.

Cloning and expression of SARS-CoV-2 spike protein

The SARS-CoV2 spike ectodomain amino acids 17-1213 and the RBD-SD1 domain aa 319-519 (based on QHD43416)[24] were cloned and expressed in the human cell line Expi293 (Thermo Fisher Scientific, Germany) (details **Supplementary Methods 1**).

Testing for PF4/heparin-reactive and platelet-activating immunoglobulin G antibodies

For screening of all sera of the COVID-19 cohorts and the patients with VITT, we used an IgG-specific anti-PF4/heparin ELISA, with antibody binding measured using a secondary anti-human IgG antibody, as described.[25] PF4 derived from platelets and recombinant PF4 were obtained from Chromatec, Greifswald. Optical density (OD) results <0.5 units were considered negative, ≥0.5<1.0 weak-positive, and OD≥1.0 strong-positive.

We performed platelet activation assays using purified, washed platelets from healthy volunteers, as described,[5] using patient sera, or the respective purified anti-PF4 IgG fractions (**Supplementary Methods 2**) with and without addition of PF4 (10 µg/mL) (Chromatec, Greifswald, Germany). Unfractionated heparin (100 IU/mL, final) was added to evaluate inhibition of antibody- and PF4-dependent platelet activation. Platelet activation was judged positive if at least two of 3 donor cells aggregated within 30 minutes.[26,27]

Binding studies of affinity-purified anti-PF4 IgG to SARS-CoV-2 S-1 domain, receptor-binding domain, full-length spike protein, PF4 and PF4/heparin complexes

We identified sera testing positive for anti-PF4/heparin antibodies from two patient groups, (a) patients with COVID-19 disease (only a minority tested positive), and (b) patients with VITT (all tested positive). These sera were assessed for reactivity against spike protein antigens using the following targets: SARS-CoV-2 full-length spike protein and the receptor-binding domain (RDB-SD1) (both assessed using in-house ELISAs), and a commercially-available CoV-2 ELISA (recombinant S1-domain; EI 2606-9620 G; EUROIMMUN Medizinische Labordiagnostika AG, Lübeck, Germany). Anti-PF4 and anti-PF4/heparin affinity-purified IgG fractions of 14 VITT patients (all with documented thromboembolic events) were used in a 1:20 dilution (detailed description in **Supplementary Methods 3**).

COVID-19 Patient Cohorts

A total of 222 COVID-19 patients were enrolled from five prospective registries from University Medical Centers in Munich (CORKUM, WHO trial ID DRKS00021225), Freiburg (WHO trial ID DRKS00021206), Tuebingen (approval by the local ethics committee 240/2018BO2), Greifswald (DRKS-ID: DRKS00023770) and Bari, Italy (approval by the local ethics committee, ID Number: NP 4463). Patients between the age of 4 months to 88 years with at least 1 ml available serum or citrate anticoagulated plasma and positive PCR testing of SARS-CoV-2 in nasopharyngeal swabs were enrolled. Registries began recruiting patients at varying start dates ranging from February 2020 until October 2020. Patient characteristics are summarized in **Table 1**; registries are described in detail in the **Supplementary Methods 4, Supplementary Tables SM1 and SM2**.

Ethics

All studies on COVID 19 patients have been approved by the local institutional review board/independent ethics committee. All patients (or their representatives) provided written informed consent.

The use of whole blood and washed platelets from healthy adult individuals, and the use of blood from VITT patients was approved by the Ethics Committee of the University Medicine Greifswald. All volunteers gave written informed consent in accordance with the Declaration of Helsinki. All experiments were carried out in accordance with the approved guidelines.

Data Sharing

Please contact Andreas Greinacher andreas.greinacher@med.uni-greifswald.de for data presented in this manuscript. Data will be made available to researchers upon reasonable request.

Results

Similarities in human PF4 and the SARS-CoV-2 spike protein structures

PF4 and the SARS-CoV-2 spike protein show sequence homologies (**Supplementary Table SR1A and SR1B**). The spike protein (6vxx.pbd: 323-335) displays high similarity to two consecutive epitopes within PF4 (6-21/23-43), although the spike epitope resembles a planar configuration, and the PF4 structure is more of a pleated sheet (see **supplementary Figure S1A**). This motif in PF4 is involved in binding heparin (see **supplementary Figure S1B,C**).[28] The identical structure is expressed by PF4variant1. (further details in **Supplementary Results SR1**)

VITT patient cohort

We evaluated sera from 24 patients with confirmed VITT developing after the first dose of the ChAdOx1 nCoV-19 COVID-19 vaccine. As expected in the context of an early primary immune response, the sera of these (recently-vaccinated) 24 VITT patients contained mostly weakly to moderately binding IgG to the S1 sequence and the RBD sequence of the spike protein, with somewhat higher levels of ELISA reactivity using the full-length spike protein. In contrast, all VITT sera showed binding to PF4 and PF4/heparin complexes, most with high reactivity (OD>2.5; **Figure 1A**). As our PF4 preparation contains 1.6 - 2% PF4variant1 (as determined by proteome analysis), we also tested nine of the VITT sera with recombinant PF4 (which lacks PF4variant1), which showed the same results **(supplementary Figure S2**). This excludes reactivity being caused by binding only to PF4variant1. The slightly stronger signal with PF4/heparin complexes is most likely explained by a higher amount of PF4 being available on the plates when they are coated with PF4/heparin complexes instead of single PF4 molecules. The variable reactivity of VITT sera with PF4 alone is probably caused by conformational alteration of PF4 upon coating to the plastic surface, which affects binding of a subset of VITT antibodies. This is consistent with the known variable sensitivities of anti-PF4/polyanion assays for VITT-related anti-PF4 antibodies, which presumably are related to differences in test characteristics including how the antigen is coated.[29-31]

No serological cross-reactivity of purified anti-PF4 antibodies from VITT patient serum with recombinant SARS-CoV-2 spike protein

To determine whether reactivity of VITT sera to the spike protein and to PF4 was due to cross-reactivity of the same antibodies binding to both proteins, or whether antibodies with different specificities were present, we affinity-purified the anti-PF4 antibodies from 14 VITT patient sera (from which sufficient amounts were available), using both PF4 and PF4/heparin complexes. As shown in **Figure 1A**, right panel, the affinity-purified antibodies once again bound to PF4 and PF4/heparin complexes, indicating successful affinity purification. The weaker reactivity compared to the original sera results from loss and dilution of antibodies during purification. However, we cannot exclude that by affinity purification a fraction of PF4-specific antibodies in VITT serum was lost because of failed recognition of the chemically-modified biotinylated PF4. This may include antibodies that are cross-reactive with spike protein. We therefore also affinity purified antibodies using complexes of 30% biotinylated PF4, 70% native PF4 and heparin. Affinity-purified antibodies strongly activated platelets in the presence of PF4 (**Figure 1B**). However, none of the affinity-purified antibodies bound to any of the SARS-CoV-2 spike protein constructs (**Figure 1A**).

COVID-19 patient cohorts

From five medical centers, sera from a total of 222 COVID-19 patients (125 males, 97 females; median age, 55 years [range, 4 months to 88 years]) were evaluated in the IgG-specific PF4/heparin ELISA. Nineteen of 222 (8.6%) patients tested positive, with 13 yielding a result between OD 0.500 and <1.000, and 6 testing between OD 1.000 to <2.000) (**Table 1**). There was no correlation between WHO severity score of COVID-19 disease and antibody reactivity by PF4/heparin ELISA (**Figure 2A**).

Sera from all 19 patients who tested positive in the anti-PF4/heparin ELISA were tested in the platelet activation assay in the presence of heparin and of PF4, respectively, to judge heparin- and PF4-dependent platelet activation. The PF4-enhanced washed platelet activation assay is currently the most sensitive test in our laboratory for detecting VITT platelet-activating antibodies and is more sensitive than the flow cytometry whole blood assay we have recently described (data not shown).[32]

Under reaction conditions previously shown to result in strong VITT patient serum-induced platelet activation (PF4, 10 µg/mL), we found that 4/19 sera showed weak to moderate PF4-dependent platelet activation (lag time, median 15 minutes, range 10 to 30 min); in contrast, none of these sera showed platelet activation in the presence of 0.2 anti-factor Xa U/mL low-molecular-weight heparin. For 10/222 patients (4.5%), thromboembolic complications were reported (six patients with pulmonary embolism, one patient with stroke, two patients with portal vein thrombosis, one thrombosis of unknown localization). Nine of these 10 patients tested negative by PF4/heparin ELISA. Only one serum was reactive with OD>1.0; for this patient a pulmonary embolism was reported. However, none of these ten sera, including the ELISA-positive patient with pulmonary embolism, induced platelet aggregation in the platelet activation test, regardless of whether heparin or PF4 was added. Moreover, there was no difference in platelet counts in patients with and without thrombosis (**Figure 2B**).

Discussion

Vaccination against the SARS-CoV-2 spike protein with ChAdOx1 nCov-19 or COVID-19 Vaccine Janssen can induce antibodies that cause marked PF4-dependent platelet activation with resulting thrombocytopenia and unusual thromboses. Most individuals after SARS-CoV-2 vaccination and patients with COVID-19 express antibodies against the spike protein.[33-36] Structural analysis of both the spike protein and PF4 indicated potential cross-reactive epitopes. Although the identified linear sequences are similar, the depicted three-dimensional configurations differ slightly between spike protein and PF4. The relevant structure "323-335" on PF4, however, is a flexible loop, which can also fold-on-demand, e.g., when a high affinity antibody binds. Moreover, this loop in PF4 has already been shown to change its conformation when heparin binds.[28] This raised the concern that SARS-CoV-2 vaccination might trigger formation of anti-spike protein antibodies that cause VITT by cross-reacting with PF4, resulting in pathogenic PF4-mediated platelet activation.

Using purified recombinant spike protein, purified PF4, and affinity-purified anti-PF4 antibodies from sera obtained from VITT patients, we found no cross-reactivity between the platelet activating anti-PF4 antibodies with the spike protein of SARS-CoV-2. Affinity-purified anti-PF4 antibodies from sera of VITT patients strongly bound in the PF4/heparin ELISA, and induced strong PF4-dependent platelet activation. However, they neither bound to full-length spike protein, the S1 domain, nor the RBD-S1 domain. In contrast, most sera tested from COVID-19 patients contained antibodies that strongly bound to the spike protein, but not to PF4 or PF4/heparin complexes (**Figure 1A**). This indicates that the immune responses against both proteins, PF4 and spike, are independent of one another.

There are some limitations to our experiments. Affinity purification might preferentially select for a fraction of antibodies from VITT patient sera that retains antibody binding to biotinylated PF4 but which lacks cross-reactivity against spike protein, if these “lost” antibodies recognize an epitope covered by biotin on PF4. To address this possibility, we t additionally purified antibodies using complexes composed of 30% biotinylated PF4 and 70% native PF4; the resulting affinity purified antibodies also did not bind to any of the spike protein constructs tested. Another limitation is that we could not test spike protein after it has been cleaved by furin or TMPRSS2. Although unlikely, we cannot exclude that such cleavage would induce conformational changes allowing binding of anti-PF4 antibodies. A further limitation is that we could not purify anti-spike protein antibodies from COVID-19 patients (to assess in a reverse fashion for cross-reactivity against PF4), due to limited material.

The reason why VITT patients produce high-titer platelet activating anti-PF4 antibodies is currently unknown. PF4 and the related protein PF4variant1[37] have gained major attention in autoimmunity.[38-40] Conceivably, predisposition to autoimmunity might lead to disruption of self-tolerance against PF4.

In parallel with these studies, we assessed whether COVID-19 patients who usually have a strong immune response against the spike protein develop anti-PF4 antibodies similar to those found in VITT patients, potentially explaining thrombosis associated with COVID-19. However, in a combined analysis of five patient cohorts comprising 222 COVID-19 patients with variable clinical disease severity (according to the WHO COVID-19 severity score), we found no evidence for an association between anti-PF4/heparin IgG and thromboembolic complications in COVID-19 patients. The frequency of anti-PF4/heparin IgG detectable by ELISA was 8.6%. This number was even lower than that observed in a prospective study in non-COVID-19 intensive care unit patients (17.2% anti-PF4/heparin IgG ELISA positive and 5.5% platelet activation test positive).[41] None of the COVID-19 patients showed heparin-dependent platelet-activating antibodies, while the frequency of PF4-dependent platelet-activating antibodies was only 1.9% (4/222). Moreover, the reactivity of these four COVID-19 patients' sera were all weak compared to the generally strong reactivity seen with VITT sera (lag times, median 15 minutes versus <2-5 minutes, respectively).

Overall, COVID-19 patients with and without anti-PF4 antibodies or PF4-dependent platelet-activating antibodies showed similar clinical characteristics. In particular, none of the patients with PF4-dependent platelet-activating antibodies developed thrombosis. In our multicentre COVID-19 patient cohorts, thromboembolic events occurred in 4.5% of patients, with no cerebral vein thrombosis or splanchnic vein thrombosis reported. Only one patient with thrombosis was reactive in the PF4/heparin ELISA, but that patient's serum did not activate platelets, either in the presence of heparin or PF4. This indicates that thrombotic events in COVID-19 patients are not typically associated with the presence of the same anti-PF4 platelet-activating antibodies identified in vaccinated people who develop VITT. This does not exclude that on rare occasions COVID-19 patients could develop prothrombotic PF4-dependent antibodies that activate platelets, but this remains to be established. However, such a phenomenon would be independent of the immune response against the SARS-CoV-2 spike protein, which is highly prevalent in this patient population.

Taken together, based on our findings it is unlikely that the intended immune response against the SARS-CoV-2 spike protein itself induces severe VITT by inducing anti-spike protein antibodies cross-reacting with PF4 (or PF4variant1). This information is critical for further risk-benefit assessment of the ongoing large vaccination programs. Our study indicates there is no apparent need to change the SARS-CoV-2 spike protein antigen target for the vaccination strategy to curtail the pandemic.

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Author contributions:

AG, KS and TT developed the concept of the study; AA and SR produced the recombinant spike protein constructs; JM, MM, JCH, and OTK developed the COVID-19 Registry of the LMU Klinikum (CORKUM) and organized sample and data transfer; DD, AL, SR collected reported cases from cohort Freiburg and organized sample and data transfer; MPG, KALM collected reported cases from cohort Tuebingen and organized sample and data transfer; CS, MN and KH collected reported cases from cohort Greifswald and organized sample and data transfer; GL, AV, AF, PL, and AS collected reported cases from cohort Brescia and organized sample and data transfer; RM provided the structural analysis and comparison of the PF4 and spike protein; RP and JW performed the IgG affinity purification, in vitro antibody cross-reactivity experiments, analyzed the data, and prepared the figure; AG, KS, and TEW analysed the data; AG, KS, JM, TEW, TT wrote the manuscript. All authors have critically revised and approved the final version of the manuscript.

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Giuseppe De Palma, University of Brescia, provided samples and cared for study patients.

Konstanze Aurich, Dept. of Transfusion Medicine, University Medicine Greifswald, reviewed the results and the manuscript, and organized cohort laboratory investigations.
Khalil Eldebuch, Dept. of Transfusion Medicine, University Medicine Greifswald, helped with testing of the affinity purified antibodies in the functional test.

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Competing interests

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All authors declare their competing interests in the COI forms.

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Figure legends

Figure 1: Immunoglobulin G antibodies in COVID-19 patients and VITT patients against anti-SARS-CoV-2 spike protein and PF4.

A) Shown are the individual results of optical density (OD) of sera tested by enzyme-linked immunosorbent assay (ELISA), median with interquartile range are indicated by red lines. Sera of COVID-19 patients (n=20), VITT patients (n=24) and PF4-affinity-purified (✳) IgG or PF4/heparin affinity-purified (○) IgG from VITT sera (n=14) are shown. The 14 sera used for affinity purification of anti-PF4 IgG are indicated by green filled symbols. All sera and the respective affinity-purified anti-PF4 immunoglobulin G fractions were tested against SARS-CoV-2 S1 domain, RBD-SD1 domain, spike full-length ectodomain, PF4, and PF4/heparin complexes. Sera of COVID-19 patients reacted with the spike protein and its S1 and RBD domains but not with PF4 or PF4/heparin complexes. VITT sera reacted with spike protein epitopes and PF4 but strongest with PF4/heparin complexes, while the affinity-purified anti-PF4 antibody fraction reacted with PF4 and PF4/heparin complexes but not with the spike protein or its S1 and RBD-SD1 domains. Negative controls (n=15) gave all negative results (**supplementary Figure S3**). The positive controls in the experiment with affinity purified antibody for binding of antibodies to the S1 domain, the RBD domain, and the spike protein where positive (data not shown for figure clarity).

B) As control that the affinity purified anti-PF4 antibodies could still activate platelets, we incubated 75 µL washed platelets in Tyrodes buffer with PF4 (10 µg/mL) and added 10 µL of the affinity purified antibodies, which reacted as the original serum. Results of 14 affinity purified antibody fractions are shown.12 showed strong platelet activation in the presence of PF4. Two antibody fractions still reacted positive by PF4/heparin ELISA but no longer activated platelets, most likely due to too low antibody yield after affinity purification.

Figure 2: PF4/heparin ELISA OD and platelet count in COVID-19 patient

A) Results of PF4/heparin ELISA (OD) are given in relation to the WHO Severity Score of COVID-19 disease of 222 COVID-19 patients. Cut off (ELISA) = 0.5 OD. The 10 patients who developed thrombosis are indicated by open circles. 212 patients without thrombosis are indicated by solid symbols. There was no correlation between WHO severity score of COVID-19 disease and reactivity on the PF4/heparin ELISA.

B) Platelet counts in COVID-19 patients with and without thrombosis.

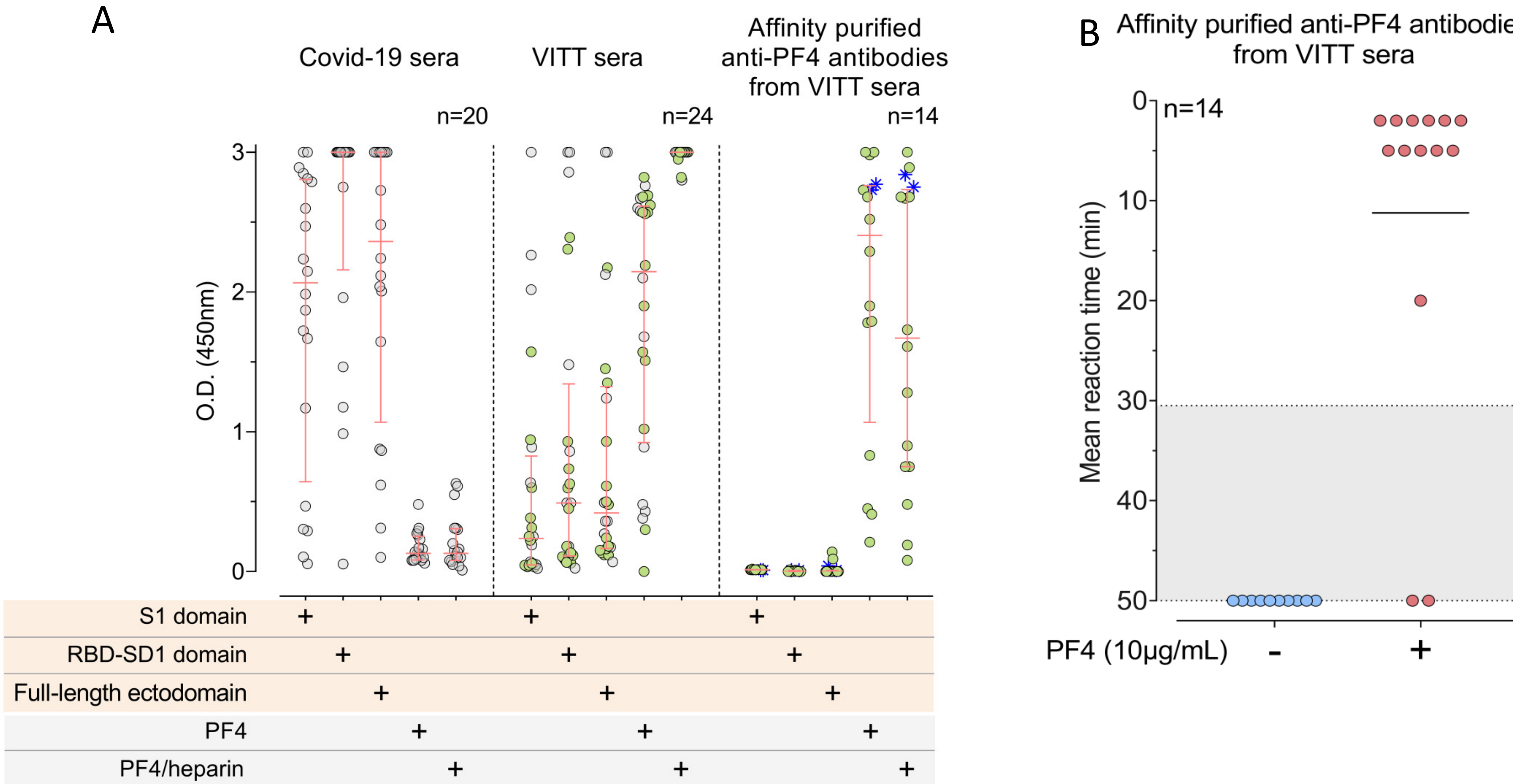
Table 1: Patient characteristics, data collected from 5 university hospitals: Freiburg (n=42), Munich (n=55), Tuebingen (n=32), Greifswald (n=32), Bari (n=61)

	COVID-19 patients without thrombosis	COVID-19 patients with thrombosis*
Number of patients, n=222 (%)	212 (100)	10 (100)
- female, n (%)	93 (43.8)	4 (40.0)
- male, n (%)	119 (56.2)	6 (60.0)
age, median (range)	55 (0.4-88)	55 (23-84)
<60, n (%)	146 (68.9)	6 (60.0)
≥60, n (%)	66 (31.1)	4 (40.0)
Outpatient care, n (%)	61 (28.8)	0
hospitalization, n (%)	151 (71.2)	10 (100)
- General ward (% of all patients)	122 (57.5)	8 (80.0)
- Intensive care unit (% of all patients)	29 (13.7)	2 (20.0)
WHO COVID-19 Score, n (%)		
- 1-3	87 (41.0)	3 (30.0)
- 4-5	105 (49.5)	5 (50.0)
- 6-9	18 (8.5)	2 (20.0)
- 10	2 (0.95)	0
Interval from symptoms to blood drawing, n (%)		
- Day 0-10	115 (54.2)	4 (40.0)
- Day 11-20	55 (25.9)	5 (50.0)
- Day 21-50	37 (17.5)	1 (10.0)
- >50 days	5 (2.4)	
Platelets at time of blood drawing, Gpt/L		
- mean (range)	239 (24-769)	223 (82-364)
Patients with platelet count, n (%)		
- >150 Gpt/L	178 (83.9)	7 (70.0)
- >100 -150 Gpt/L	24 (11.3)	2 (20.0)
- 50 ≤100 Gpt/L	7 (3.3)	1 (10.0)
- <50 Gpt/L	1 (0.47)	0
- Missing data	2 (0.94)	
Heparin treatment (at least 5 days) before blood drawing, n (%)	32 (15.0)	3 (30.0)
- missing data	2 (0.94)	0
PF4/heparin ELISA, n (%)		
- OD<0.5	194 (91.5)	9 (90.0)
- OD≥0.5 <1.0	13 (6.1)	0
- OD≥1.0	5 (2.4)	1 (10.0)
Heparin dependent platelet activation, (sera with PF4/heparin ELISA OD≥0.5), n (%)		
- Negative	18 (8.5)	10[#]
- Positive	0	0
PF4 dependent platelet activation, (sera with PF4/heparin ELISA OD≥0.5), n (%)		

- Negative	14 (6.6)	10#
- Positive	4 (1.9)	0
Outcome, n (%)		
- Survived	206 (97.2)	10 (100)
- in hospital deaths	6 (2.8)	0

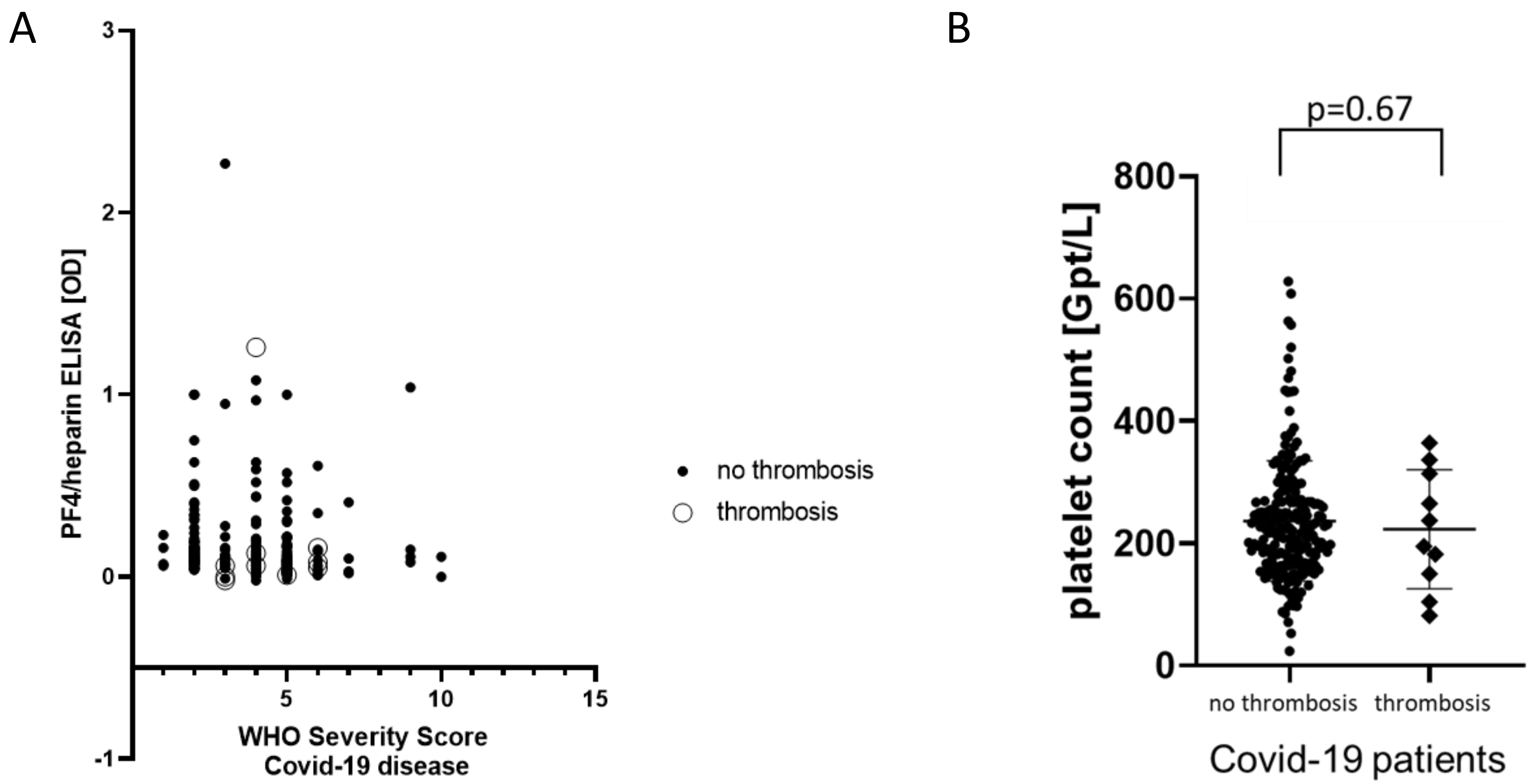
*thrombosis localization: 6x LAE; 1x stroke; 2x portal vein, 1x unknown: #all sera from patients with thrombosis were tested independently of the PF4/heparin ELISA result.

Figure 1



A
Covid-19 sera
VITT sera
Affinity purified anti-PF4 antibodies from VITT sera
n=20
n=24
n=14
O.D. (450nm)
S1 domain
RBD-SD1 domain
Full-length ectodomain
PF4
PF4/heparin
B
Affinity purified anti-PF4 antibodies from VITT sera
n=14
Mean reaction time (min)
PF4 (10µg/mL)
-
+

Figure 2



A
3
2
1
0
-1
PF4/heparin ELISA [OD]
5
10
15
WHO Severity Score
Covid-19 disease
no thrombosis
thrombosis
B
p=0.67
800
600
400
200
0
platelet count [Gpt/L]
no thrombosis
thrombosis
Covid-19 patients